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Chemotherapy-Induced Expression of αB-Crystallin in Neuroblastoma

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Since αB-crystallin is known to be expressed in glial tissues of human brain and neuroectodermal tumors, the αB-crystallin content of neuroblastomas, may be related to the degree of glial or neuronal differentiation. The αB-crystallin content of 73 neuroblastomas, was determined by enzyme immunoassay. The concentration of αB-crystallin was examined in light of neuroblastoma prognostic factors. Neuroblastomas from patients who received chemotherapy (n = 23) contained higher concentrations of αB-crystallin than those from patients who did not receive chemotherapy (n = 50) ($P > 0.05$). There was a statistically significant difference in αB-crystallin concentrations in advanced stage patients who received preoperative chemotherapy ($P < 0.01$). Immunohistochemistry demonstrated αB-crystallin expression in the nerve-like fibers and a few ganglion-like cells. Staining was not apparent in the less differentiated cells in the tumor cell nest. αB-crystallin may play a role in the response to cellular stress in neuroblastoma. Med. Pediatr. Oncol. 29:11–15, 1997.

Key words αB-crystallin; differentiation; neuroblastoma; prognosis; chemotherapy; enzyme immunoassay

INTRODUCTION

Expression of αB-crystallin, a major structural protein of the vertebrate eye lens, also is observed in extra-lenticular tissues that include the central nervous system, skeletal muscle, and kidney. Iwaki et al. [1] have shown that αB-crystallin is expressed by glial cells and also is present in astrocytic components of neuroectodermal neoplasms by immunocytochemical studies [2]. The physiologic significance of αB-crystallin expression outside the lens is unclear. Recently, similarities in amino acid sequence between αB-crystallin and small heat shock proteins (HSPs) have been revealed [3,4]. Klemenz et al. [5] and Kato et al. [6] have reported the induction of αB-crystallin expression in response to heat or chemical stress in NIH 3T3 fibroblasts and in glioma cell lines (U373 MG and U118 MG).

Neuroblastoma is derived from the neural crest and is the most common solid malignant tumor of childhood. This tumor sometimes demonstrates morphologic differentiation to neuronal or Schwannian cells either spontaneously or following treatment. Shimada et al. [7] have reported that the degree of ganglionic and Schwannian differentiation is related to the prognosis of this disease. We hypothesized that the αB-crystallin content in neuroblastomas may be related to the degree of glial or neuronal differentiation. In this study we determined the αB-crystallin content of neuroblastomas by enzyme immunoassay.

MATERIALS AND METHODS

Tissues

Tumor tissues were obtained intraoperatively from 73 Japanese patients with neuroblastoma. There were 39 males and 34 females aged 5 days to 13 years (mean 2.2 years). Diagnoses included undifferentiated neuroblastoma (50) and ganglioneuroblastoma (23). All samples were frozen rapidly and stored at −80°C until assayed. Patients were classified into five categories (I-24, II-10, III-16, IV-16, IVs-7) according to the pretreatment staging criteria of Evans et al. [8]. Eight of 16 patients with stage III neuroblastoma and 12 of 16 patients with stage IV neuroblastoma received intensive preoperative chemotherapy with cyclophosphamide (CPA), vincristine (VCR), pirarubicin (THP-ADM), and cisplatin. Tumor tissues from these patients were obtained within 7 months (mean 89 days, 17–198 days) after the initiation of chemotherapy. Postoperatively, 28 of 41 patients with classified as stages I, II, and IVs received adjuvant chemotherapy with CPA, VCR, and THP-ADM for 3–12 months. Nine stage III patients without disease recur-

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TABLE I. Concentration of αB-Crystallin in Neuroblastoma

	n	All cases[b]	n	no chemotherapy	n	Chemotherapy[d]
Ganglioneuroblastoma[c]	23	185.6 ± 47.2[a]	10	63.9 ± 50.5	13	279.2 ± 63.8
Undifferentiated Neuroblastoma	50	40.0 ± 12.2	40	41.8 ± 14.8	10	32.7 ± 16.4
Total[e]	73	84.2 ± 17.5	50	48.1 ± 14.4	23	172.0 ± 44.5

[a]mean ± SE, ng/mg soluble protein
[b]ganglioneuroblastoma vs undifferentiated neuroblastoma = $P < 0.05$
[c]no chemotherapy vs chemotherapy = $P < 0.01$
[d]ganglioneuroblastoma vs undifferentiated neuroblastoma = $P < 0.01$
[e]no chemotherapy vs chemotherapy = $P < 0.05$

rence received chemotherapy postoperatively for 6–24 months. Fifteen of 24 patients with distant metastases or recurrences underwent bone marrow transplantation after achieving a complete clinical remission. Survival time was determined from the start of treatment. The diagnosis was confirmed histologically in each case.

Preparation of Tissue Extracts

Tissues were homogenized with a Polytron-type homogenizer at 0°C in 10 volumes of 50 mM Tris-HCl (pH 7.5) containing 5 mM EDTA. Each homogenate was centrifuged at 4°C (125,000 × g for 40 min), and the supernatant was used for the αB-crystallin assay. Each extract was diluted 10-fold with buffer, and 0.5 ml aliquots of the diluted sample underwent immunoassay in duplicate.

Preparation of αB-crystallin and Antibody Production

Human αB-crystallin was purified from skeletal muscle as described by Kato et al. [9]. Antibodies were raised in rabbits with human αB-crystallin from pectoral muscle or the C-terminal decapeptide of αB-crystallin as an immunogen. Antisera were purified as previously described [10].

Enzyme Immunoassay

Human αB-crystallin was assayed by a sandwich-type immunoassay as described by Kato et al. [10]. The system consisted of a solid phase (polystyrene ball) with immobilized purified antibodies to human αB-crystallin and the same antibodies labeled with β-D-galactosidase from *Escherichia coli.* Purified human αB-crystallin was used to generate a standard curve. Results were expressed as ng per mg of soluble protein.

Protein concentrations in crude extracts were determined by a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Richmond, CA). This kit used protein-dye binding [11] and bovine serum albumin as a standard.

Statistical Analysis

The Dunnett multiple comparison test, a nonparametric method, was used for the analysis of differences between groups. The event-time distributions were estimated by the product limit method of Kaplan and Meier. Differences between event-time distributions were tested for statistical significance by using the logrank test. Analysis were performed using the statistical software Fisher.

RESULTS

Concentration of αB-crystallin in Neuroblastomas

The mean concentration of αB-crystallin in 73 neuroblastomas was determined (Table I). A significantly higher concentration of αB-crystallin was found in ganglioneuroblastomas as compared to neuroblastomas ($P < 0.01$). However, tumors from patients who received chemotherapy (n = 23) contained higher concentrations of αB-crystallin (172.0 ± 44.5 ng/mg protein, mean ± SE) than those from patients who did not receive chemotherapy (n = 50) (48.1 ± 14.4 ng/mg protein, mean ± SE)($P < 0.05$). Therefore, in tumors from patients who did not receive chemotherapy (n = 50), there was no statistically significant difference between undifferentiated neuroblastoma and ganglioneuroblastoma. These concentrations were similar to the mean concentration of αB-crystallin in ganglioneuroma (64.1 ± 46.0 ng/mg protein, n = 6), a benign tumor similar to neuroblastoma. The mean duration from the beginning of preoperative chemotherapy to the time of surgical resection was 86.4 days (17–198, SE = 11.1). There was no correlation between the concentration of αB-crystallin and the duration of preoperative chemotherapy. The mean concentration of αB-crystallin in 11 neuroblastomas that underwent preoperative chemotherapy for a period longer than 70 days (172.3 ± 67.6 ng/mg protein, ±SE), was similar to that of 12 neuroblastomas that underwent preoperative chemotherapy for a period shorter than 71 days (171.7 ± 61.6 ng/mg protein, ± SE). However, two neuroblastomas from patients that underwent preoperative chemotherapy for a period less than 30 days contained relatively low concentrations (1.36 and 3.43 ng ng/mg protein, respectively) of αB-crystallin as compared to the overall mean concentration (172.0 ± 44.5 ng/mg protein).

TABLE II. Concentration of αβ-Crystallin and Clinical Stages of Neuroblastoma

State	n	All cases[b]	n	No chemotherapy##	n	Chemotherapy[b]
I	24	68.0 ± 29.8[a]	24	68.0 ± 29.8	0	—
II	10	40.9 ± 22.1	10	40.9 ± 22.1	0	—
III	16	72.4 ± 28.4	8	20.4 ± 11.5	8	124.4 ± 50.5
IV[c]	16	187.3 ± 59.7	4	10.9 ± 9.8	12	246.1 ± 72.3
IVs	7	6.7 ± 3.2	4	9.8 ± 5.2	3	2.6 ± 1.8

[a]mean ± SE, ng/mg soluble protein
[b]IV vs IVs = $P < 0.05$
[c]no chemotherapy vs chemotherapy = $P < 0.05$
##$P > 0.05$

Concentrations of αB-crystallin and the Clinical Stage of Neuroblastoma

The mean concentrations of neuroblastoma αB-crystallin were determined in patients at different disease stages (Table II). In tumors from 50 patients that did not receive chemotherapy, there was a trend toward decreasing αB-crystallin concentrations with advanced stages. However, when all cases (n = 73) were considered the mean value of αB-crystallin was the highest in stage IV cases (n = 16, 187.3 ng/mg soluble protein) because tumors from stage IV patients who received chemotherapy had very high concentrations (n = 12, mean 246.1 ng/mg soluble protein). However, preoperative chemotherapy did not effect the level of αB-crystallin in stage IVs cases (n = 3, mean 2.6 ng/mg soluble protein). There was a statistical significance in concentrations of αB-crystallin between stage IV and IVs patients who received preoperative chemotherapy ($P > 0.05$).

Correlation Between αB-crystallin Concentrations and Patient Survival

Stages I and II patients did not receive preoperative chemotherapy and were all tumor-free at 2 years. Thirteen of 32 advanced stage (III and IV) patients had died at 2 years from the beginning of treatment. In patients who received preoperative chemotherapy αB-crystallin concentrations were higher in tumors from patients that were alive at 2 years after treatment (254.1 ± 67.3 vs 71.5 ± 44.4 ng/mg soluble protein, mean ± SE, $P < 0.05$). In patients who received preoperative chemotherapy, the correlations between the concentration of αB-crystallin and cumulative survival of patients with neuroblastoma were determined by the Kaplan-Meier method. Patients were evaluated according to whether their tumor concentration of αB-crystallin was greater or lesser than 50 ng per mg of soluble protein (Figure 1). αB-crystallin of >50 ng per mg of soluble protein was associated with a better survival ($P = 0.045$). However, in patients who did not receive preoperative chemotherapy, 6 of 12 neuroblastomas contained concentrations of αB-crystallin lower than 1 ng/mg protein (data not shown) and αB-crystallin concentrations did not differ in relation to patient outcome.

Immunohistochemical Localization of αB-crystallin in Neuroblastomas

There was diffuse expression of αB-crystallin in the nerve-like fibers and a few ganglion-like cells in ganglioneuroblastomas from patients who received preoperative chemotherapy. This staining was homogenous, and positive staining was not apparent in less differentiated cells in the tumor cell nest.

DISCUSSION

Recently, many investigators have reported the presence of αB-crystallin in extra-lenticular tissues [1,10,12–14]. In normal human brain αB-crystallin is found in glial tissues [1,15]. Iwaki et al. [2] have reported the expression of αB-crystallin in astrocytic elements of neuroectodermal tumors by immunostaining methods and in glioma cell lines, but not in neuroblastoma cell lines, by immunostaining and Northern blotting.

In the present study we showed the expression of αB-crystallin in neuroblastomas. There were increasing αB-crystallin concentrations in neuroblastomas from patients (stage III and IV) that had undergone preoperative chemotherapy, although there was a large variation in concentrations (0.35–755 ng/mg protein) among neuroblastomas.

Neuroblastoma is one of the most aggressive childhood tumors. Stage, age, histologic differentiation and N-myc amplification are regarded as the most significant prognostic factors. We examined the relationship between the expression of αB-crystallin and these prognostic factors for significant correlations. Most patients of advanced stage (III, IV) received preoperative chemotherapy immediately after diagnosis. It was rather difficult to obtained a sufficient number of samples from untreated patients with advanced disease stages. We therefore analyzed samples from patients with and without treatment before surgery. We confirmed that high

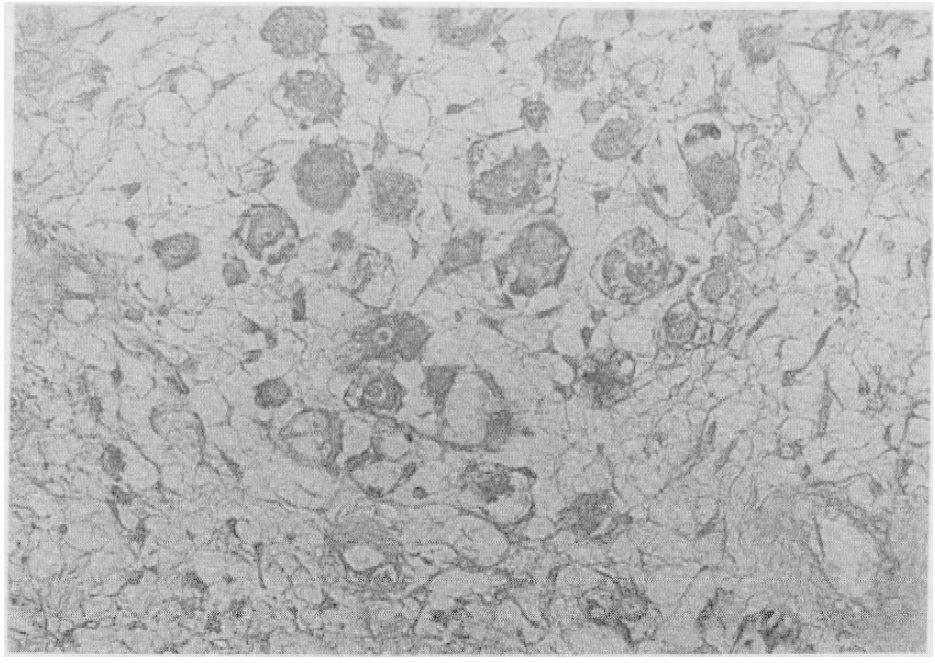

Fig. 1. Correlation between the tumor levels of αB-crystallin and overall survival rate of children with neuroblastomas, calculated by the Kaplan-Meier method. There are statistical significance between two groups ($P < 0.05$). □ = αB-crystallin concentration >50 ng/mg soluble protein, ● = αB-crystallin concentration < 50 ng/mg soluble protein.

concentrations of αB-crystallin were associated with pathologic differentiation when the total number of cases (n = 73) were examined. However, there was no relation between high concentrations of αB-crystallin and pathologic differentiation in neuroblastomas from the subset of patients that did not receive preoperative chemotherapy. Neuroblastomas from patients who received chemotherapy contained higher concentrations of αB-crystallin than those from patients that did not receive chemotherapy, especially in advanced disease stages (III, IV) (197.4 vs 17.2 ng/mg protein, $P < 0.01$). Our results indicate that chemotherapy may be one of the factors increasing αB-crystallin concentrations in neuroblastoma. There were no statistical correlations between the duration of preoperative chemotherapy and the concentration of αB-crystallin. However, tumors treated for shorter than 1 month contained low concentrations of αB-crystallin. These results indicate that increasing of αB-crystallin concentrations may be a response of tumor tissues to preoperative chemotherapy. However, in stage IVs, that is a favorable stage as well as stage I or stage II, the mean concentrations of αB-crystallin (n = 3, mean 2.6 ng/mg soluble protein) in tumors from patients who received chemotherapy was lower than that (n = 4, mean 9.8 ng/mg soluble protein) from patients who did not receive chemotherapy.

The primary structure of αB-crystallin is highly homologous to the mammalian small heat shock protein HSP28 [3,4]. Inaguma et al. [16] have reported that the concentrations of αB-crystallin and HSP28 increased after heat treatment of rat glioma cells. In neuroblastoma cell lines we saw that the expression of αB-crystallin was very low without heat treatment (n = 4, mean 1.3 ng/mg protein) when compared to surgical sample, and heat treatment caused an increase in αB-crystallin expression (n = 3, mean 41.3 ng/mg protein) by approximately 30–50 fold (data not shown). Ciocca et al. [17] have reported that elevated HSPs concentrations were associated with doxorubicin resistance in human breast cancer cells. We can therefore speculate that chemotherapy may induce a continuous stress response in neuroblastomas that results in the overexpression of HSPs. Although αB-crystallin expression in the untreated group was less than in the treated group, there was a trend to decreasing αB-crystallin concentrations with stages. This result is compatible with the report of Ungar et al. [18] concerning stages and HSP27 expression in neuroblastomas. Seymour et al. [19] and Tetu et al. [20] also have reported that overexpression of HSP27 in tumors (breast cancer or malignant fibrous histiocytoma) was associated with longer survival. These results may indicate that lower concentrations of small HSPs may be a component of an environment conducive to tumor cell proliferations. Ungar et al. [19] have investigated the relationship between HSP27 concentrations and N-myc amplication in untreated neuroblastomas. In our series, αB-Crystallin concentrations were not associated with biochemical (neuron specific enolase, telomerase, or HSP28), genetic (N-myc

or Trk A), or clinical (patient age or tumor location) factors.

In conclusion, we can speculate that αB-crystallin may play a role in the response to cellular stress in neuroblastomas and that neuroblastomas with high expression of αB-crystallin may not be conducive to tumor growth.

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